

THE EFFECT OF COPPER (II) ON SURVIVAL, RESPIRATION,
AND HEART RATE IN THE COMMON BLUE MUSSEL,
MYTILUS EDULIS

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Although copper in trace quantities is required by living organisms, small increments above the required level are highly toxic. Copper looms most importantly in the Crustacea and Mollusca where it forms a part of hemocyanin, an oxygen transport pigment, about which much remains unresolved (Wilbur and Yonge, 1965). Hemocyanin has been identified in all classes of the Mollusca except the Bivalvia which does not appear to have a functional oxygen-carrying pigment (Morton, 1958). Copper is an important constituent of both tyrosinase and cytochrome oxidases and as such presumably has a universal distribution in all classes. Prytherch (1931) showed that copper is required for the attachment and metamorphosis of the oyster, *Crassostrea virginica*. The physiological changes accompanying toxic Cu(II) levels have not been deeply examined. These effects are of current relevance in northeastern New England where a heavily mineralized coastal belt is being returned to active copper mining with discharge of mineral rich washings into coastal waters. In 1968 mining began at Cape Rosier (46° 21' N, 68° 47' W) on Penobscot Bay, Maine and new operations are planned for 1972 in nearby Blue Hill Bay.

MATERIALS AND METHODS

Collection and maintenance of animals

Animals for respiratory work, heart rate studies, and copper concentration studies were collected at Lamoine, Maine, between May 1970 and January 1971, from water ranging in temperature from -1 to 15° C. Animals were returned to the laboratory and kept in 10 gallon tanks, continuously aerated, which were maintained at 10° C. Carboys of water were also taken since the use of artificial sea water (Instant Ocean, Aquarium Systems, Inc.) was found to be unsatisfactory. Those animals in the synthetic mixture consistently showed a higher degree of valve closure than those in natural sea water. Redistilled water was added to maintain the specific gravity at 1.025, and the pH was checked frequently. It varied from 8.0 to 8.2.

Copper measurements

The method chosen for extraction of copper was that of Riley and Sinhaseni (1958). The reagent for extraction and spectrophotometric determination of copper in sea water was 2:2'-diquinolyl in *n*-hexanol. The color of the cuprous

diquinoyl complex was stabilized by addition of hydroquinone. 2:2'-diquinoyl reacts with copper in the cuprous state and in order to ensure that all copper is in this state, hydroxylamine hydrochloride is added. Absorbances were determined in a Bausch and Lomb Spectronic 20 spectrophotometer using a 1 cm light-path cell. Measurements were made at 540 nm and compared with a standard curve. The procedure was periodically checked by adding known amounts of Cu(II) to standard and experimental samples and determining recovery of the added Cu(II). Riley and Sinhaseni report a coefficient of variation of this method of 2.5% with sea water containing 0.027 mg/l copper. As employed one measures total copper.

Survival studies

Laboratory studies were carried out in an effort to determine the toxicity threshold for *M. edulis*. Duplicate glass tanks were set up for each of four copper concentrations (0.5, 0.3, 0.2 and 0.1 mg/l). Each tank contained 10 liters of sea water and 100 animals 1 to 2 cm in length. All tanks were well aerated, kept at 10° C, and the animals were not fed. It should be noted that this was a static system, and concentrations, as indicated were initial concentrations only. No attempt was made to maintain the levels of copper after the animals were introduced to the tanks. Ideally one would use a continuous flow system of fresh unfiltered sea water with a constantly maintained concentration of the metal as was done by Pringle *et al.* (1968). On the other hand, Raymont and Shields (1962) have concluded that although a constant flow is desirable in "long term" studies, there is no significant difference in the toxicity threshold (for *Mya*) as measured in the constant flow and static systems.

The copper salt chosen for stock solution was copper(II) chloride dihydrate. Working with *Nereis virens*, Raymont and Shields (1962) point out that while there may be slight differences in the toxicities of various cupric salts, the threshold toxicity is approximately the same.

Warburg respirometry

Respiration of *M. edulis* has been shown to vary with body size and temperature (Read, 1962), salinity (Schlieper, 1929; Maloeuf, 1938), seasonal change (Krüger, 1960), and reproductive state (Bruce, 1926). Reproductive state of the animals was not established in this study but animals were paired randomly from collections made on the same date.

The ratio of tissue wet weight to shell length was measured and found to be remarkably constant and essentially linear from 4–8 cm where the ratio was 1.2 g per cm. None of the animals were fed, and a starvation curve (holding time τ_s , endogenous respiration) was established. Animals were not used in respiration studies after a holding period of five days, although they did not show significant respiratory declines until the 10th day in the holding tanks. All measurements were at 10° C in a refrigerated Warburg respirometer and animals were selected to fit this equipment. Thirty animals, all 1.3 cm $\pm$ 0.1 cm were dried at 100° C for 18 hours. The dry weights (minus shell) had a range of 0.0117 g to 0.0129 g with a mean of 0.0121 g. No attempt was made to eliminate bacterial contamina-

tion since most anti-bacterial steps would involve the possibility of some toxicity effects on the mussels. Were the contents of each flask to be a successful bacterial culture then the bacterial contribution to total oxygen consumption would be an exponential effect. Beginning, in terms of biomass, several orders of magnitude below the molluscan biomass the bacterial oxygen contribution should not be perceptible over the time course of these experiments and should it intrude it should intrude exponentially. Control runs remain linear over the course of these experiments and tank water controls show no significant respiration vis-a-vis distilled water thermobarometers. Others have faced this same problem (Rottauwe, 1958; Read, 1962). The respiratory changes parallel the changes in other parameters measured and we are satisfied that the respiration measured does not include a significant bacterial contribution. The current investigation is based on a reading every 10 minutes and a mean based on one hour. Standard Warburg flasks (approximately 20 ml) contained four animals (1.3 cm each) in a volume of 4.05 ml of sea water.

Oxygen consumption may be looked upon as a convenient measure of energy transformation. The change in *M. edulis* from aerobic respiration may take place not only when poor conditions prevail but also, without apparent reason, in a well aerated environment (Rottauwe, 1958). It is important to use only those animals which appear to be in the same condition in the holding tanks. Only those with opened valves and extended mantles were chosen for this study. Observations on the condition of the animals were kept during all Warburg runs, and when any of the control animals closed down their valves, the readings from that flask were excluded from computation of the mean endogenous respiratory rate. Shaking stimulates shell closing.

None of the Warburg flasks were shaken to promote oxygen exchange across the air-water interface. According to Rottauwe (1958) above 50% air saturation at 19° C the rate of oxygen uptake of *M. edulis* becomes independent of the partial pressure. Thus without determining per cent air saturation of the sea water in the Warburg flasks, the assumption is being made that, during the two hour runs, the water is more than 50% saturated. This seems probable since all runs in this work are at 10° C, and at this temperature more oxygen is dissolved in the water than at 19° C. Furthermore, the greatest uptake recorded for a single flask is 30 μ l/hr, and the filtering carried on by the animals inevitably promotes some gaseous exchange. The uptake of control animals remained linear over a period of several hours, even though at the maximum rate of oxygen consumption, 100% of the oxygen originally in the sea water (30.0 μ l at air saturation) is used during the first hour of the experiment. On rare occasions, during preliminary work, the molluscs remained open while being shaken. The values obtained during these runs were the same as those found in the unshaken runs used in data collection.

Copper uptake studies

Attempts were made to measure copper uptake in the mussels and to determine the time course of the uptake. A tank system was set up with the same ratio of sea water to animal tissue as there was in the Warburg flasks. Each tank contained two liters of sea water (0.3 mg/l Cu(II)) and 44 animals, each 5.3 cm in length. Four animals were removed at 12, 22, 36, 48 and 72 hours and each

mussel was then analyzed for copper. Control animals from sea water tanks containing no copper were also analyzed.

Detoxification mechanisms

Warburg runs were made as indicated with Cu(II) levels at 0.3 and 0.5 mg/ml. After 90 minutes the original organisms were removed and replaced with two new mussels, the flasks closed off, and oxygen measurement resumed. The procedure was repeated four times at each of the two Cu(II) levels. Depression of the respiratory rate would then be a measure of the toxicity remaining in the sea water.

A second procedure was also employed. Whole *M. edulis* were homogenized in sea water for five minutes using a standard teflon pestle tissue grinder in a glass vessel. They were then made up to 10% wt/vol with additional sea water. Of this homogenate, 0.2 ml was then added to 0.3 and 0.5 mg/l copper-sea water solutions whose volume was equivalent to the volumes used in the Warburg runs.

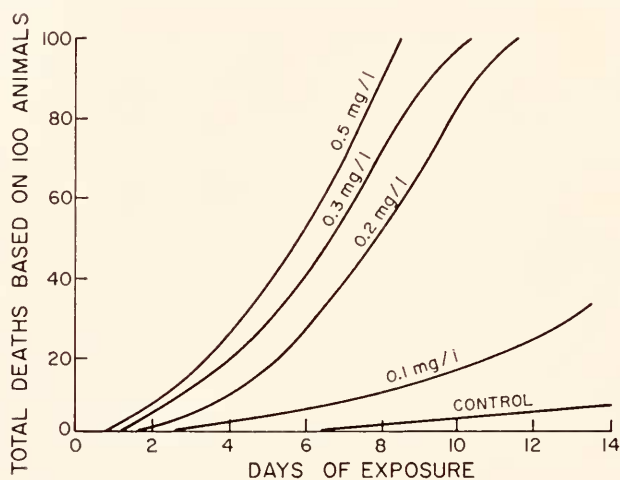


FIGURE 1. Toxicity experiment, 10° C.

The mixture was then incubated at 10° C in air for 90 minutes, centrifuged in a clinical centrifuge at 1800 g for 15 minutes, and the supernatant used as the incubation medium for the standard Warburg procedures already described. Each supernatant was tested for toxicity four times by this standard Warburg procedure. Depression of respiration would indicate the persistence of toxicity.

Heart rate studies

Heart rates were obtained by cutting small (2 cm × 2 cm) windows in the left valve directly over the beating heart. All rates were measured at 10° C and only ventricular beats were counted. Schlieper (1955) has shown that when the shell of *M. edulis* closed, the heart rate may be reduced fourteenfold. The decreased heart rate as noted by Schlieper was not observed although there were frequent periods during which the heart completely ceased beating. In the present

study several control animals were held in the closed position by placing rubber bands around the shells. No difference was noted in the heart rates of these animals or in the frequency or duration of non-beating periods. These periods did not appear to be related to valve closure and only periods of beating were included in calculation of heart rates during copper treatments.

RESULTS

Where two Cu(II) treatments are compared the t test at 5% level is employed while, with more complex comparisons, analysis of variance is employed and, where F is significant, Duncan's means discrimination test at the 5% level is the test of significance.

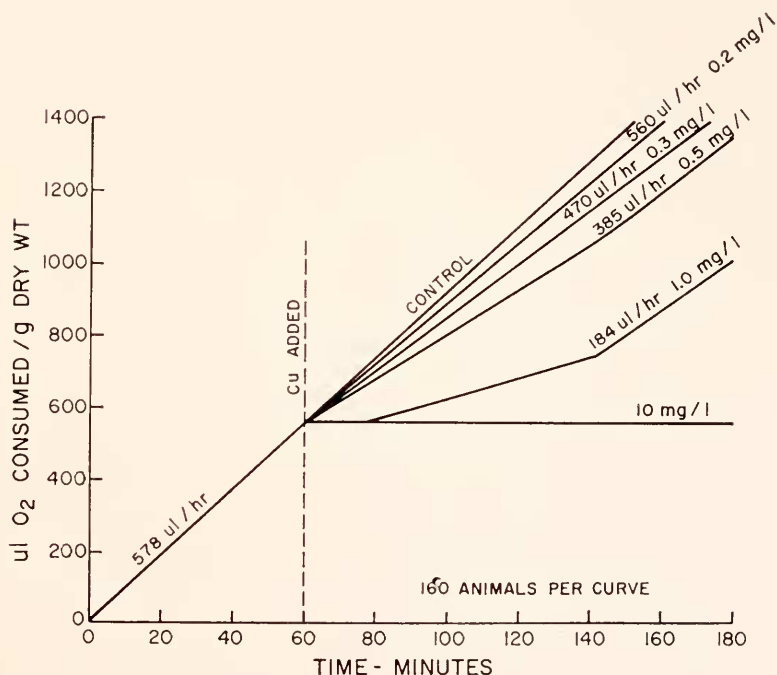


FIGURE 2. The effect of Cu(II) on *M. edulis* respiration at 10° C.

From Figure 1 it can be seen that the threshold toxicity appears between 0.1–0.2 mg/l. After seven days, 55% of the animals kept at 0.2 mg/l (initial concentration) were dead, whereas only 5% died after an equal period of time in 0.1 mg/l. Controls showed 1% mortality over the same time period. Comparable results were obtained by Marks (1962) working with *M. edulis*, Pringle *et al.* (1968) working with *Mya arenaria*, and Raymont and Shields (1962) working with *Nereis virens*. These authors set the toxicity threshold of copper for their respective animals at about 0.2 mg/l.

Results of respiratory studies are expressed as the mean of twenty runs (80 animals) at each concentration (Fig. 2). In all cases copper was added from the

side arm of the flask at 60 minutes and the final volume of sea water in the flask was 4.05 ml with the indicated concentration of copper. In the case of controls, sea water was added from the side arm at 60 minutes. Studies were made with final copper concentrations of 10.0, 1.0, 0.5 and 0.2 mg/l.

Figure 2 shows the effects of copper on the respiration of *M. edulis*. Analysis of variance on the change in respiration (between $t = 80$ min and $t = 140$ min) shows significance at the 1% level. A Duncan's comparison of the mean changes in respiration for each of the plots shows no significance between the control and the 0.2 mg/l respiratory values. Depressions at the higher concentrations of copper are all significant.

At 10 mg/l the valves of the animals closed immediately after copper addition and remained closed for at least 15 hours, at which time the study was discontinued. At 1.0 mg/l the animals shutdown for approximately 15 minutes, after which

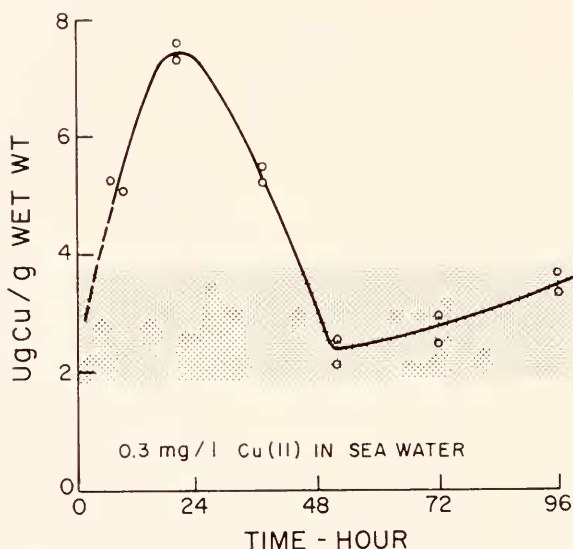


FIGURE 3. Cu(II) accumulation in *M. edulis* at 10° C.

the valves opened and the rate of oxygen consumption became 184 μ l/hr. Approximately 90 minutes after addition of the copper there was a sudden increase in respiration until the rate approached the rate of oxygen consumption for the controls (578 μ l/hr/g). At 0.5 mg/l there was no initial shutdown, but a recovery similar to that just described was apparent. At 0.3 mg/l there was neither a shutdown nor apparent recovery, and finally, at 0.2 mg/l copper, the change in respiration as measured over 2 hours at 10° C is not significantly different from control values. This data shows a dose response for the effect of copper on *M. edulis* respiration. Assuming a linear relationship, the equation for the change in respiration at 10° C over one hour as a function of copper concentration is:

$$\mu\text{l O}_2 \text{ consumed/g (dry wt)/hr} = 578 - 394 (\text{Cu II conc. in mg/l}).$$

The results of copper uptake studies are indicated in Figure 3. Copper concentrations peaked at 22 hours exposure and then declined to the normal range.

The results of both methods employed in detoxification studies were similar. Animal respiration was within normal limits after treating sea water-copper solutions as previously indicated with either live animals or heat killed homogenate.

The heart rates of the points on the graph (Fig. 4) are the mean heart rates of eight animals at each concentration. Two standard deviations are indicated about each point. Copper was added only after regular beating was established. Controls showed regular beating for 24 hours. No change in heart rate at 0.2 mg/l copper was noted, but at higher concentrations a general dose response is clearly apparent within one hour.

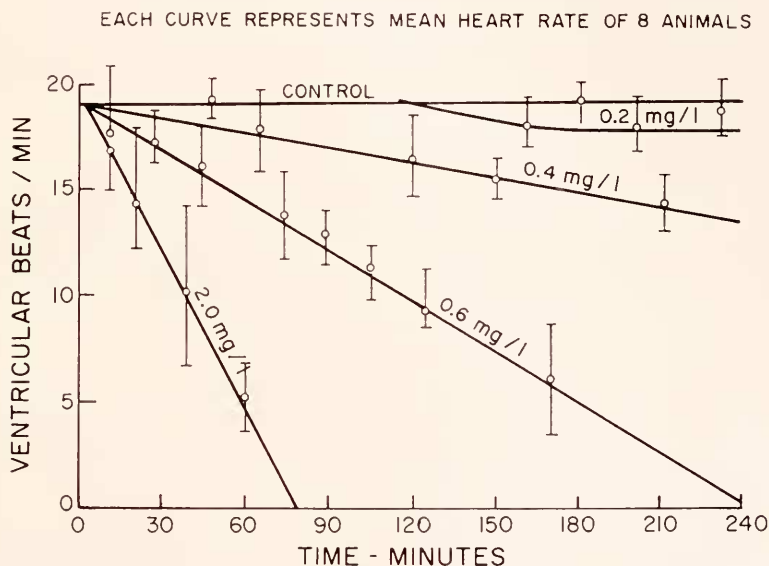


FIGURE 4. Effect of Cu(II) on *M. edulis* heart rate at 10° C.

DISCUSSION

In the sea, normal concentrations of copper lie between 0.01 mg/l in coastal waters (Atkins, 1932), and 0.003 mg/l in the open sea (Goldberg, 1963). Little is known of the copper cycle, and the distribution of this metal in the open sea, although Riley (1937) suggests that in deeper oceanic waters concentrations are significantly higher than at the surface. The Federal Water Quality Administration Report (1970) sampled 30 stations in Penobscot and Blue Hill Bays during 1967 and 1968 and reported water values ranging from 0.04 mg/l-0.37 mg/l around Cape Rosier area with the Blue Hill area twelve miles to the northeast ranging from 0.02-0.04 mg/l. Our own values, 12 determinations in each case, obtained in October 1970 give a mean of 0.0191 mg/l total copper in the water near the mine site and 0.0212 for our control area which was at Lamorne, Maine (44° 27' N, 68° 17' W), 25 miles to the northeast and marked by the same mineralization but lacking a mining history. Our values show no significant

current difference between the two sites. *Mytilus edulis* was collected from the two sites and those from the Cape Rosier area contained 6.567 mg/kg (wet weight) copper, and the Lamoine controls 2.987 mg/kg. These differences are significant and consistent. Populations at Cape Rosier are sparse but apparently healthy and the control area shows only a higher population density. While the water levels of copper may fluctuate it is apparent that molluscs show the cumulative effects.

The literature is replete with studies showing that molluscs, polychaetes, and plankton have the ability to concentrate copper and other metals several thousandfold above ambient water levels. (Rose and Bodansky, 1920; Severy, 1923; Vinadogray, 1953; Chipman, Rice and Price, 1958; McFarren, Campbell and Engel, 1961; Galtsoff, 1964; Brooks and Rumsby, 1965; Pringle, Hissong, Katz and Mulawka, 1968). These papers indicate that all bivalves tested are proficient copper concentrators with *Crassostrea virginica* which reaches 19 mg/kg (Kopfler, 1966) being the most efficient and showing higher metal concentrations as one proceeds northward.

It is difficult to reconcile this data with exposure data in toxicology studies where *M. edulis* was killed in 12 hours by 0.55 mg/l copper (Ingols, 1955) and survived only 3–4 days following immersion of from 0.5–3.0 days in 0.14 mg/l copper (Clarke, 1947). The length of time that *M. edulis* could survive depended to some extent on its ability to keep its shell closed. In our toxicity studies 0.3 mg/l Cu(II) killed 55% of *M. edulis*, following an exposure of seven days. In Warburg studies, Cu(II) caused respiratory depression which became less obvious as the Cu(II) level declined. At the lower copper levels where this depression was clearly significant there was complete respiratory recovery despite continuous exposure. The live organisms had neutralized the copper effect. The alteration that occurred with time was not an adaptation of the organism but a change in the solution in some way. This is shown by the fact that new live organisms, added to the originally toxic solution after previously exposed organisms showed recovery, did not go through an adaptation stage. The solution in which they were placed was simply nontoxic. Using a heat-killed *M. edulis* homogenate one can also produce this detoxification of a Cu(II) solution.

All of these preceding data could be explained if the copper inside an organism were in some form that is nontoxic. One of the simplest ways for the Cu(II) to be effectively removed from the solution would be its passive binding with available organic ligands within the organism. One is inclined to this solution of the problem by the fact that a homogenized heat-killed extract can duplicate the action of the live organism.

During uptake studies at 0.3 mg/l Cu(II), the immediate response of mussels was to secrete copious amounts of mucous and this appeared consistently. Attempts to analyze this mucous, and the sea water into which it was released, failed due to formation of a stable emulsion during copper extraction procedures. However, Koringa (1952) found that cations can be absorbed on the mucous of the gills of *Crassostrea virginica*. Increased mucous secretion and subsequent binding may be another method of detoxifying sea water-Cu(II) solutions.

Clearing of the initial influx of Cu(II) is apparent from the uptake study (Fig. 3). It may be that the return to control copper levels is by clearing the

initial internal copper (II) by transfer *via* excreted ligands, for example, by increased mucous secretion from the gills of the animal. When animals are placed in 0.3 mg/l copper, increased mucous production is observed. A second possibility is that copper is transferred to metabolic wastes and removed *via* the feces. Since live animals collected at Cape Rosier showed tissue levels (6.5 mg/kg) nearly as high as those attained in this experiment (7.5 mg/kg) one would not necessarily expect such levels to be lethal to *M. edulis*. Although animals taken in the Cape Rosier Region appear to be able to maintain 6.5 mg/kg in their tissues and remain in a viable condition, the animals in this experiment have been subjected to ionic copper.

This may indicate differences in the state of the copper in the two situations. From our data this is probably due to organic binding in the environment. Furthermore, since the toxicity studies show that death is caused in 100% of the animals exposed to 0.3 mg/l Cu(II), it is concluded that the lethal damage is done during the first 36 hours and is essentially irreversible.

When free Cu(II) is added to an ecosystem it must be presumed that organic binding occurs and that ingestion of such organically bound Cu(II) does not provide toxic levels of free Cu(II). Some organisms will be damaged by the free Cu(II) as it is bound since the initial binding presumably is the toxic step but, subsequently, members of the food chain may accumulate extraordinary levels of the organically bound copper without apparent effect. Total copper in a marine ecosystem is probably not the pertinent parameter of toxicity.

SUMMARY

1. The effects of Cu(II) on survival heart rate, and respiration in *M. edulis* have been determined.
2. Free Cu(II) is toxic to *M. edulis* causing respiratory and cardio-vascular depression and a toxicity threshold of 0.2 mg/l has been demonstrated.
3. Live animals can detoxify solutions of limited Cu(II) content.
4. Heat-killed homogenates are also effective in detoxifying solutions of limited Cu(II) content.

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